RESEARCH COMMUNICATION

Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor

James S. DAVIDSON,* Ian K. WAKEFIELD and Robert P. MILLAR

MRC Regulatory Peptides Research Unit, Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa

Desensitization of gonadotropin release by the pituitary gland in response to gonadotropin-releasing hormone (GnRH) agonists has clinical applications in the treatment of gonadal-hormone-dependent disorders. We therefore investigated possible desensitization of inositol phosphate (IP) responses of GnRH receptors. No short-term homologous desensitization of the IP response to GnRH was observed in either α T3 gonadotrope cells line or GH₃ cells transfected with GnRH receptor cDNA. The absence of homologous desensitization is unusual among G-protein-coupled receptors, and may be due to the absence of a C-terminal

cytoplasmic tail, a unique feature of the GnRH receptor. Several potential protein kinase C phosphorylation sites which might mediate heterologous desensitization are present on the GnRH receptor. In both α T3 cells and GnRH-receptor-transfected Cos-1 cells, activation of protein kinase C by pretreatment with phorbol ester caused a 35–53 % decrease in the IP response to GnRH. However, phorbol ester also inhibited guanosine 5'-[γ -thio]triphosphate-stimulated IP production in permeabilized Cos-1 cells, suggesting that this inhibition is mediated at a post-receptor site.

INTRODUCTION

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is released in pulsatile fashion, giving rise to a corresponding pulsatile release of pituitary gonadotropins. The pulsatility is important in maintaining the responsiveness of the pituitary, as continuous exposure to GnRH agonists leads to a transitory stimulation of gonadotropin release which is invariably followed by inhibition [1,2]. This desensitization of gonadotropin release by agonists has found wide clinical application in the treatment of gonadal-hormone-dependent disorders [1,3] and is therefore of considerable interest. However, the mechanisms mediating gonadotrope desensitization have not been clearly elucidated.

The GnRH receptor (GnRHR) is a member of the GTP-binding protein-coupled receptor (GPCR) family [4], and activates phospholipase C via the G_q group of G-proteins [5]. Inositol trisphosphate production triggers a complex Ca²⁺ signalling process [6], leading to exocytosis of luteinizing hormone [7]. Gonadotrope desensitization could be mediated at any stage in this sequence of signalling events, and/or at the level of gonadotropin synthesis or processing.

GPCRs display two types of desensitization. Homologous desensitization refers to agonist-induced desensitization which is specific for the receptor activated by the agonist. In contrast, when exposure to an agonist (or other pharmacological agent) desensitizes other distinct receptors that do not interact with the agonist used; this is termed heterologous desensitization [8]. Two mechanisms have been described which are capable of mediating homologous desensitization, both of which may depend on sites in the C-terminal cytoplasmic tail of the receptor [9–13].

GnRHRs from four species have now been cloned, and all lack a C-terminal cytoplasmic tail [4,14–20]. This feature, which is unique among known GPCRs, raises the possibility that the GnRHR might not be capable of desensitization by the above

mechanisms. Indeed, we show here that the GnRHR shows no short-term homologous desensitization of its ability to stimulate phospholipase C. In addition, we show that protein kinase C-mediated heterologous desensitization of GnRH-stimulated inositol phosphate (IP) production is exerted at a post-receptor site.

MATERIALS AND METHODS

Cell culture

The α T3 gonadotrope cell line was provided by Dr. P. Mellon, University of California, San Diego, CA, U.S.A. GH₃, Cos-1 and α T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10 % fetal calf serum, in 5 % CO₂.

Transfection with GnRHR cDNA

cDNAs for the human [15] and mouse [4] GnRHRs were subcloned into the mammalian expression vector pcDNA1/Amp (Invitrogen). DNA was prepared using Qiagen columns according to the manufacturer's instructions. Cells were seeded on poly(D-lysine)-coated 12-well plates 1 day before transfection, at 1.8×10^5 cells per well. Cos-1 cells were transfected with plasmid DNA by a modification of the DEAE-dextran method [21]. Cells were washed twice with PBS, then incubated with 0.5 ml/well serum-free DMEM containing 5 μ g/ml plasmid DNA and 0.3 mg/ml DEAE-dextran for 4 h at 37 °C. This was replaced with 1 ml of DMEM containing 2 % fetal calf serum and 200 μ M chloroquine, and incubation was continued for 1 h at 37 °C. After this the cells were washed twice with PBS, and 1 ml of DMEM with 10 % fetal calf serum was added. GH₃ cells were transfected by incubating with 1 μ g/well DNA and 4 μ l/well Lipofectamine reagent (Gibco) in 0.5 ml of serum-free DMEM

Abbreviations used: GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; TRH, thyrotropin-releasing hormone; GPCR, G-protein-coupled receptor; GTP[S], guanosine 5'-[γ -thio]triphosphate; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate.

* To whom correspondence should be addressed: Department of Medicine, University of Bristol, Upper Maudlin Street, Bristol BS2 8HW, U.K.

for 5 h at 37 °C, after which 1 ml of DMEM with 10 % fetal calf serum was added.

IP production

At 1 day after transfection, cells were labelled overnight with 1 μ Ci/ml *myo*-[2-³H]inositol (Amersham) in 0.25 ml/well Medium 199 (Gibco) containing 5 % fetal calf serum, washed twice in buffer A [22], then stimulated with GnRH, thyrotropin-releasing hormone (TRH) or guanosine 5'-[γ -thio]triphosphate (GTP[S]) as indicated. LiCl (10 mM) was added at varying times in relation to GnRH, as indicated. Incubation were stopped with HClO₄/phytic acid solution, and total IPs were eluted from Dowex columns as previously described [22]. IP production was expressed as a percentage of total HClO₄-insoluble radioactivity in each well, determined by washing the cell residue three times with HClO₄ stopping solution [22], solubilizing with 0.1 M NaOH and neutralizing with 6.6 % acetic acid.

Cell permeabilization

Cos-1 cells were permeabilized with 10 μ g/ml Staphylococcal α -toxin [23], and permeabilization was monitored by the release of [2-³H]deoxyglucose as previously described [24]. Staphylococcal α -toxin (10 μ g/ml) induced the release of over 80 % of cell-associated radiolabel within 15 min (results not shown). Staphylococcal α -toxin was obtained from Dr. S. Bhakdi, Institut für Medizinische Mikrobiologie, Johannes Gutenberg Universität, Mainz, Germany.

Data points are means $\pm$ range of duplicate wells in a single experiment which is representative of three similar experiments. Error bars are omitted from Figures when smaller than the symbol.

RESULTS

Possible desensitization of the IP response to GnRH was determined by measuring the rate of IP accumulation in the presence of Li⁺ as a function of time after the onset of stimulation by GnRH. For assessment of desensitization occurring in the first few minutes of agonist exposure, α T3 cells or GnRHR-transfected GH₃ cells were preincubated with Li⁺ and then stimulated with a supramaximal concentration of GnRH. In α T3 cells the rate of IP accumulation (3.9 ± 0.6 % of total cell radiolabel/min; $n = 7$ experiments) was linear for the first 5 min (Figure 1a). After 5–10 min there was a decline in the rate, which flattened progressively at later times (results not shown). This decline was due to depletion of labelled phospholipid substrate, rather than desensitization (see below). The absence of desensitization was also observed with submaximal concentrations (1–10 nM) of GnRH (results not shown).

In GnRHR-transfected GH₃ cells, the rate of IP production was 30-fold lower than in α T3 cells, and was linear up to 10 min, the longest time period tested (Figure 1b). In contrast, the rate of TRH-stimulated IP production in GH₃ cells showed a rapid decline over the first 5 min of agonist exposure (Figure 1c). The initial rate (measured between 0 and 30 s) of TRH-stimulated IP production was 2.87 ± 1.09 % of total cell radiolabel/min, and this decreased to 0.14 ± 0.04 % measured between 5 and 10 min, a mean decrease of 94.3 % ($n = 3$). Thus the TRH receptor rapidly develops homologous desensitization, while the GnRHR does not.

The very rapid rate of GnRH-stimulated phosphoinositide hydrolysis in α T3 cells (39 ± 6.1 % of total label in 10 min; see Figure 1a) suggested that the decrease in rate seen after 5–10 min

was due to depletion of radiolabelled substrate. To circumvent this problem in α T3 cells, we performed experiments in which the rate of IP accumulation was measured following addition of Li⁺ at later times after the start of GnRH stimulation, a method which avoids depletion of radiolabel from the phospholipid pool by allowing the recycling of labelled inositol [25]. In these experiments, the rate of IP accumulation did not decrease with time up to 90 min of GnRH stimulation (Figure 2). This confirms that the decline seen in α T3 cells after 5–10 min was due to depletion of labelled phosphoinositide substrate and not desensitization.

To probe possible roles for the consensus protein kinase C phosphorylation sites on the GnRHR, α T3 cells and GnRHR-transfected Cos-1 cells were pretreated with 0.1 μ M phorbol 12-myristate 13-acetate (PMA) for 10 min to activate protein kinase C, and then stimulated with 0.1 μ M GnRH for 60 min. In mouse GnRHR-transfected Cos-1 cells, PMA treatment reduced the IP response to GnRH by 40 ± 8.7 % (mean $\pm$ S.E.M.; $n = 4$ experiments). In α T3 and human GnRHR-transfected Cos-1 cells,

Figure 1 Early time course of IP responses to GnRH in α T3 cells (a), to GnRH in mouse GnRHR-transfected GH₃ cells (b) and to TRH in GH₃ cells (c)

Cells were pre-incubated with LiCl for 10 min before addition of 1 μ M GnRH or TRH. Filled symbols, stimulated cells; open symbols, unstimulated cells. IP production is expressed as a percentage of total radiolabel in HClO₄-insoluble cellular material.

Figure 2 IP accumulation in α T3 cells following Li^+ addition at varying times after the start of GnRH stimulation

Cells were stimulated from time zero with $1 \mu\text{M}$ GnRH (●) or not stimulated (○). LiCl (10 mM) was added at 0, 30, 60 or 90 min after the onset of GnRH stimulation, as indicated. Unstimulated cells had LiCl from time zero. Reactions were stopped at 0, 5, 10 and 15 min after LiCl addition.

PMA similarly caused 35% and 53% decreases in GnRH-stimulated IP production (single experiments).

Although these results suggested that phosphorylation of the GnRHR by protein kinase C might lead to desensitization, it was also possible that the effect of PMA was mediated at a step(s) distal to the receptor which are known to be modulated by protein kinase C phosphorylation [26,27]. We therefore investigated the effect of PMA on IP generation stimulated by GTP[S] in permeabilized Cos-1 cells. Following permeabilization, Cos-1 cells were pretreated for 10 min with $0.1 \mu\text{M}$ PMA, then stimulated with $100 \mu\text{M}$ GTP[S] for 60 min. PMA treatment inhibited GTP[S]-stimulated IP production by $77 \pm 18\%$ (mean $\pm$ S.E.M., $n = 3$ experiments), indicating an effect at a post-receptor site.

DISCUSSION

We investigated possible short-term desensitization of the IP response to GnRH, utilizing both cloned and endogenous receptors. Particular attention was given to the first 5 min of agonist exposure, as very rapid desensitization can easily be overlooked, and care was taken to avoid artefacts due to depletion of radiolabelled substrate.

GnRH-stimulated IP production did not display short-term homologous desensitization, whether GnRHRs were expressed endogenously in α T3 gonadotrope cells or cloned receptor cDNA was transfected into GH_3 cells. In α T3 cells, the very high rate of phosphoinositide hydrolysis suggested that the decline in the rate after 5–10 min seen in these cells was due to depletion of radiolabelled substrate, rather than desensitization. This was confirmed using the method of Paris et al. [25] in which lithium is added at varying times after agonist, thereby avoiding substrate depletion. In addition, no desensitization was detected in GnRHR-transfected GH_3 cells, which had a much lower rate of phosphoinositide hydrolysis.

In contrast, the TRH receptor expressed in the same (GH_3) cell type showed rapid desensitization within 5 min. This rapid phase

of TRH receptor desensitization appears to have been overlooked in a previous study in which initial rates were taken over the first 10 min of stimulation [28].

Because of the possibility that desensitization of the GnRHR is cell-specific, the phenomenon was studied in two different cell types of pituitary origin. Although the lack of a necessary factor for rapid desensitization cannot be absolutely excluded, the absence of desensitization in both cell types suggests that this is likely to be an intrinsic property of the receptor.

The absence of rapid homologous desensitization is unusual among GPCRs. Many receptors coupled to phospholipase C, including the α_1 -adrenergic [29], H_1 -histamine [30], thrombin [25], $\text{P}_{2\text{Y}}$ -purinergic [31], angiotensin II [32], bradykinin [33], bombesin, substance P, muscarinic and cholecystokinin [34] receptors, have been shown to display marked homologous desensitization of their IP responses over a short time frame.

Different degrees and temporal patterns of desensitization have been reported for different phospholipase C-coupled receptors, and this variety of responses as well as methodological considerations involved in the measurement of phospholipase C activation have recently been reviewed [35]. The mechanisms mediating rapid homologous desensitization in phospholipase C-coupled receptors are not well defined, but may be analogous to the well-characterized β -adrenergic receptor and rhodopsin systems. In the β -adrenergic receptor and rhodopsin, homologous desensitization is mediated by phosphorylation of sites in the receptor's C-terminal cytoplasmic tail by specific kinases belonging to the β -adrenergic receptor kinase family [9]. Phosphorylation at these sites allows the binding of a protein (arrestin or β -arrestin) which is thought to sterically inhibit G-protein interaction [10]. In the $\alpha_{2\text{A}}$ -adrenergic receptor, which has a small C-terminal tail, it is instead the large third intracellular loop (157 amino acids) which is phosphorylated by β -adrenergic receptor kinase or a related kinase and mediates desensitization [36]. Recently it has been shown that β -adrenergic receptor kinases can phosphorylate the substance P receptor, suggesting that this mechanism of desensitization may also apply to phosphoinositide-coupled receptors [37].

A second mechanism for homologous desensitization has been described which also involves the C-terminal tail. In several GPCRs, domains within the tail are required for rapid receptor internalization [11–13].

In the light of this, it is of interest that the GnRHR does not display short-term homologous desensitization, since it lacks a C-terminal tail [4], and does not have a long third intracellular loop, which appears to have taken over the desensitization function in the α_2 -adrenergic receptor [36].

The desensitization of gonadotropin release which is observed following continuous exposure to GnRH agonists *in vivo*, and which is the basis for many of the therapeutic applications of the peptide [1,3], may be mediated at several levels in the signal transduction cascade: (a) GnRH receptor number down-regulation, due to internalization, increased degradation or decreased synthesis, (b) uncoupling of receptors from G-proteins, (c) modulation of signalling components such as G-proteins, phospholipases, Ca^{2+} channels and pumps, (d) decreased responsiveness of exocytotic regulatory proteins to Ca^{2+} , (e) depletion of releasable gonadotropin pools, and (f) synthesis of gonadotropins with low biological activity. The time course of *in vivo* desensitization (hours to days), as well as the results of the present study, suggest that *in vivo* desensitization to GnRH is mediated by mechanisms other than short-term desensitization of the IP signalling response.

Nevertheless, it may seem surprising that a receptor participating in a physiological system which desensitizes so markedly *in*

vivo shows no short-term desensitization. However, the absence of desensitization may not be out of keeping in a pulsatile system, which functions in a binary (on/off) manner. In such systems, the physiological response is frequency-modulated and the receptor can function as a simple amplifier of the frequency-encoded signal. In fact, it may be inappropriate for frequency-modulated systems to display desensitization over a time-frame which is short compared with the pulse duration, since this would limit the amplitude of the pulse. In the particular case of the gonadotrope, which needs to respond maximally over a period of hours during the ovulatory luteinizing hormone surge, short-term desensitization would be inappropriate, and the truncated GnRHR may have evolved to accommodate this requirement.

The inhibitory effect of phorbol ester treatment on GnRH-stimulated IP production suggested that protein kinase C might mediate heterologous desensitization by phosphorylation of the GnRHR, which has several protein kinase C consensus phosphorylation sites [4]. However, experiments in which the receptor was bypassed and G-proteins were directly stimulated with GTP[S] demonstrated that the inhibitory effect of PMA is most likely mediated by phosphorylation of a downstream component (such as a G-protein or phospholipase C) rather than at the receptor itself. A possible locus of inhibition by PMA is phospholipase C- β 1, which is known to be phosphorylated by protein kinase C, resulting in impaired coupling to G_q [27].

In conclusion, experiments designed to investigate the early time course of signal transduction by the GnRHR revealed the absence of any short-term homologous desensitization. The results also suggest that protein kinase C-mediated heterologous desensitization of GnRH-stimulated IP production occurs at a post-receptor site.

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